
Ethidium analogues with improved resolution in the dye-buoyant density procedure*

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ABSTRACT

Analogues of ethidium chloride with large quaternary side chains have been synthesized and evaluated for improved resolution in the dye-buoyant density system for the separation of open and closed circular DNA. These compounds are similar to propidium which differs from ethidium by the replacement of an ethyl group by a methyldiethylaminopropyl group. The new analogues contain a triethylamino group attached to a methylene chain with 3, 5 or 7 carbons. With PM2 DNA the observed separation for propidium is 1.97 times that of ethidium and the new dyes show separations relative to ethidium of 2.27, 2.66 and 2.77. A correlation is established between the mass of the dye component and the observed separation which is rationalized on the basis of the four component thermodynamics describing this system.

INTRODUCTION

The dye-buoyant density procedure for the detection and isolation of closed circular DNA¹⁻³ has proven to be a remarkably useful and durable technique. One of the advantages of this procedure compared, for instance, to gel electrophoretic techniques, is that the position of a band at equilibrium in the centrifuge tube can be understood on the basis of equilibrium thermodynamics⁴ and the topological properties of the closed circular DNA in question.⁵⁻⁹ The primary limitation of the technique is the limited resolution obtained for closed circular DNA's which differ only slightly in their topological properties (e.g., superhelix density or length of a topologically unconstrained segment). A previous study¹⁰ of the use of analogues of ethidium as the "dye"

component in this procedure demonstrated that propidium provides an increase in this resolution by a factor of about 1.7 to 1.9 relative to ethidium. The reason for this improved resolution was not known at that time.

In the present study a series of new ethidium analogues are tested in the dye-buoyant density system. These compounds are higher chain homologues of propidium with the common structural feature $N(5)-(CH_2)_n-N(C_2H_5)_3$ where N(5) is the phenanthridinium ring nitrogen and $n = 3, 5$ or 7 . Resolution of closed and open circular PM2 DNA is found to be greater with these dyes than with propidium. These new data in combination with the results obtained in Jerome Vinograd's laboratory¹⁰ establishes an empirical, nearly linear, relationship between the observed DNA band separation and the mass of the dye component. This empirical result is rationalized on the basis of the four-component thermodynamic theory of Bauer and Vinograd⁴ and provides a basis for predicting the increased resolution to be expected with higher homologues.

MATERIALS AND METHODS

Table 1 lists the ethidium analogues to be discussed in this paper. The new compounds designated TEAP, PENT and HEPT were synthesized from 3,8-dinitro-6-phenylphenanthridine via the dicarbethoxyamino derivative in analogy with the synthesis of propidium.¹¹ The phenanthridine nucleus was quaternized with 1,3-dibromopropane, 1,5-dibromopentane or 1,7-dibromoheptane, reacted with triethylamine and converted to the free diamine by sulfuric acid hydrolysis. The products were analyzed by TLC, NMR and elemental analysis. The details of the synthesis of these and other ethidium analogues are given elsewhere.^{12,13} Ethidium bromide and propidium diiodide were purchased from Calbiochem.

PM2 closed circular DNA was used for all of the buoyant density experiments to be described. An initial sample was kindly provided by Jerome Vinograd. Later experiments were performed with DNA extracted from PM2 phage using the method of Espejo *et al.*¹⁴ The closed circular PM2 DNA was nicked by irradiation with x-rays or with light from a 500 W slide projector in the presence of ethidium or an analogue.¹⁵⁻¹⁸

Optical grade cesium chloride was obtained from Harshaw Chemical Co. All buoyant density experiments were carried out in 0.01 M phosphate buffer, pH 6.8.

A Beckman Model L5-50 Ultracentrifuge was used with either an SW50.1 or SW56Ti rotor with cellulose nitrate centrifuge tubes. The rotor speed was 40,000 rpm and the temperature was 20°C. The tubes were removed at intervals and photographed under ultraviolet illumination (Ultraviolet Products UVL-21 Blak-Ray lamp). A Nikon F2S photomic camera with a 55 mm f/3.5 Micro Nikkor lens was used with Kodak EHI35 High Speed Ektachrome film (ASA 160). The resulting 35 mm slides were processed with a color Xerox copier to produce 6" by 9" pictures for measurement. A small plastic ruler was used as a distance standard.

Two kinds of filter arrangements were found to be useful. In the first arrangement the Blak-Ray lamp was further filtered with a Hoya U330 glass filter and two Corning glass filters (LP39 and LP47) were placed between the centrifuge tubes and the camera.¹² In this case the final filters block all the excitation light and the tubes photograph red as they appear to the eye. In the second arrangement the only filter is an Ultraviolet Products Contrast Filter (J-344) placed between the tubes and the camera. This filter transmits a small amount of blue light from the Blak-Ray lamp (410 to 440 nm) which results in color contrast in addition to intensity contrast (see Fig. 1).

The centrifuge tubes were also photographed against a white background in room light to record the dye gradient.

RESULTS

The principal results of this study are shown in Table 1 and Fig. 1. The enhancement factor χ given in Table 1 is defined as the ratio of the separation to that observed for ethidium. The value of the band separation Δr (and therefore the value of χ) depends on a variety of experimental

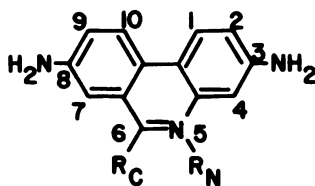


Table 1. Ethidium Analogues and the Relative Separation of PM2 DNA Components

R_C	R_N	M.W. ^a	Name ^b	χ^c
C_2H_5	C_2H_5	302		0.70 ^d
\varnothing	CH_3	336		0.92 ^d
\varnothing	C_2H_5	350	ethidium(A)	1.00
\varnothing	$(CH_2)_3 N(C_2H_5)_2 CH_3$	486	propidium(B)	1.88
\varnothing	$(CH_2)_3 N(C_2H_5)_3$	500	TEAP (C)	2.21
\varnothing	$(CH_2)_5 N(C_2H_5)_3$	528	PENT(D)	2.47
\varnothing	$(CH_2)_7 N(C_2H_5)_3$	556	HEPT(E)	2.60

a. Molecular weight of chloride or dichloride salt.

b. Trivial name. The letters refer to the tubes in Fig. 1.

c. χ is the ratio of the observed separation for the dye in question divided by the separation observed for ethidium bromide in the same run.¹⁰ d. From Ref. 10.

conditions particularly the initial solution density, the dye concentration and the run time. The data given in Table 1 correspond to the conditions of Fig. 1, namely an initial density of 1.59 g/cm³, a dye concentration of 300 μ g/ml, 6 days at 40 K rpm, 20°C and a total volume of 3.0 ml in 7/16" by 2-3/8" tubes (SW56Ti rotor). All of the tubes of Fig. 1 contain the same amount of DNA.

At equilibrium the dye component forms an inverse

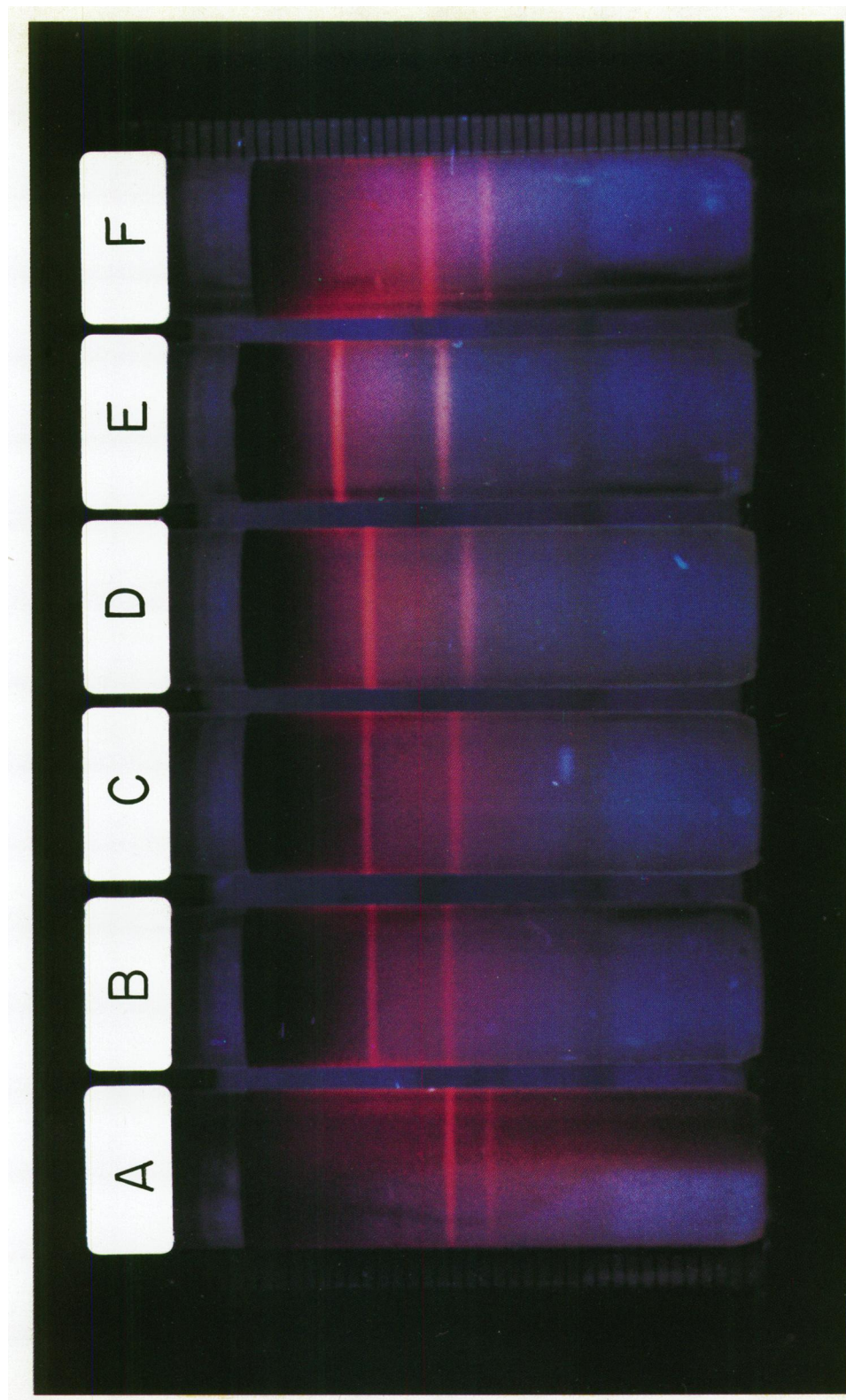


Figure 1

gradient such that a higher concentration is observed at the top of the column. This distribution is roughly exponential^{1,4}

$$C(r) \approx C_0 \exp[-\Delta r/r^*] \quad (1)$$

where

$$r^* = RT/M_4 (\bar{v}_4 \rho_0 - 1) r_0 \omega^2, \Delta r = r - r_0. \quad (2)$$

It has been assumed that $\Delta r \ll r_0$ and $\rho(r) \approx \rho_0$. See Ref. 4 for a more exact expression. The important factor is that the initial density ρ_0 or the initial dye concentration C_0 must be high enough that the dye concentration at the actual position of the upper band (form II, open circular DNA) is sufficient to effectively saturate the DNA. In our experiments a variety of values of ρ_0 and C_0 were employed to insure that the separation for ethidium and propidium was maximized.

For the series ethidium, propidium, TEAP, PENT and HEPT the dye gradient at equilibrium becomes increasingly steep since the dye mass (M_4) increases and the dye partial specific volume (\bar{v}_4) may increase. This increasing dye gradient may actually contribute to the enhanced separation observed for the larger dyes. For certain values of C_0 and ρ_0 it may happen that the DNA of the upper band (form II) is essentially saturated with dye but the dye concentration at the lower band position may be significantly lower than the effective saturation level. If this is the case, addition of dye results in movement of the form I band upward to lower density while the upper band remains fixed. This gradient enhancement effect has been previously noted for ethidium⁵ but in that case it is sufficiently small that it is not of much practical significance. For propidium and the larger dyes r^* of Eq. 2 is smaller than for ethidium and Δr is larger. The ratio of the dye concentration at the upper band to that at the lower band may be 20 or greater.

An experimental demonstration of the contribution of the dye gradient to the separation is seen in Fig. 2 which is a plot of Δr versus time for ethidium, propidium and HEPT. The increase in Δr with time reflects the contribution of the dye gradient since the dye distribution is established more slowly than the CsCl gradient or the DNA distribution.¹ (The equilibration of the CsCl gradient would cause the bands to move closer together with time). At 2 days the DNA band separation has reached 94% of its equilibrium value for ethidium, 81% for propidium and 63% for HEPT. At four days the values are 99%, 88% and 82%. TEAP and PENT have behavior intermediate to propidium and HEPT and in the expected order.

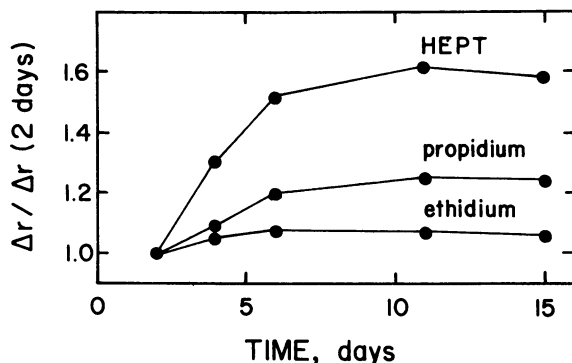


Fig. 2. The observed band separation divided by the value obtained at 2 days for ethidium, propidium and HEPT. The values of Δr at 2 days were 2.65 mm, 4.48 mm and 4.91 mm. TEAP and PENT follow similar curves between propidium and HEPT. Fig. 1 represents the 6 day points.

The values of χ reported in Table 1 are for six days of centrifugation except for the first two dyes which are smaller than ethidium where the data are for four days. For these dyes four days was sufficient to reach equilibrium to at least 95%. The value of $\chi = 1.88$ for propidium is larger than the value of 1.74 observed after four days because of the longer running time. Previous determinations of χ for propidium¹⁰ were for four days and therefore somewhat smaller

than the equilibrium values. For HEPT the values of χ observed for 2, 4, 6, and 11 days of centrifugation are 1.85, 2.30, 2.60 and 2.77 compared to the propidium values of 1.69, 1.74, 1.88, and 1.97. The ratio $\chi(\text{HEPT})/\chi(\text{propidium})$ is 1.10, 1.32, 1.38 and 1.41. It should be pointed out that although the equilibration times used for these experiments may seem excessive they may be reduced by an order of magnitude by using fixed angle or vertical rotors.

It should also be mentioned that χ is a function of the superhelix density of the closed circular DNA and is larger for low superhelix density DNAs.^{9,10} Since PM2 has an unusually high superhelix density the numbers reported here are somewhat lower than will be observed for most other DNA's.

Three other ethidium analogues have been prepared and tested in the dye buoyant density procedure. In each case $R_C = \emptyset$. The compound with $R_N = (\text{CH}_2)_5\text{N}(\text{C}_3\text{H}_7)_2$ has only one quaternized nitrogen and is not sufficiently soluble in 4 to 5 M CsCl, even at pH 5, for DNA buoyant density experiments. For $R_N = (\text{CH}_2)_3\text{N}(\text{CH}_3)_2(\text{CH}_2)_2\text{N}(\text{CH}_3)_2$ the observed separation was similar to that observed for TEAP. For $R_N = 3-(4\text{-}t\text{-butylpyrid-1-yl})\text{propyl}$ the observed value of χ was approximately 2.05. This compound is the dye component in tube F of Fig. 1 but the observed separation is considerably below the maximum value because the dye concentration (or density) is too low.

DISCUSSION

The quantity which we wish to maximize is the resolution of the dye-buoyant density system defined¹⁹ by

$$\Lambda = \Delta r / (\sigma_1 + \sigma_2) . \quad (3)$$

We have assumed that the bandwidths, σ_1 and σ_2 , are constant for all of the dyes studied since it has been shown⁴ that the effect of ethidium binding on the bandwidth is small.

We will also make the approximation that the buoyant density

difference between the closed and open DNA bands $\Delta\theta$ is proportional to Δr .^{4,9,10}

An approximate expression for the buoyant separation is¹⁰

$$\Delta\theta \approx \frac{\Delta\Gamma'(\bar{v}_3 - \bar{v}_1) + \Delta v'(\bar{v}_3 - \bar{v}_4)}{(\bar{v}_3 + \bar{\Gamma}'\bar{v}_1 + \bar{v}'\bar{v}_4)^2} \quad (4)$$

where $\Delta\Gamma' = \Gamma'_I - \Gamma'_{II}$ is the difference in the hydration of the two bands on a mass basis (grams water per gram of DNA), $\bar{\Gamma}' = (\Gamma'_I + \Gamma'_{II})/2$, \bar{v}_1 is the partial specific volume of water (approximately unity), \bar{v}_3 is the partial specific volume of anhydrous CsDNA (0.479), \bar{v}_4 is the effective partial specific volume of the dye component (1.02 ± 0.03 for ethidium chloride⁵), $\Delta v' = v'_I - v'_{II}$ is the difference in the amount of dye bound on a mass basis (grams of dye per gram of DNA) and $\bar{v}' = (v'_I + v'_{II})/2$. The numerator of Eq. 4 is accurate to the extent that the product $(\bar{v}_4 - \bar{v}_1)(\Gamma'_I v'_{II} - \Gamma'_{II} v'_I) \approx 0$. The denominator is accurate to the extent that $\Delta\Gamma'$ and $\Delta v'$ are small. If this is not true then the quantity in parentheses is to be replaced by the geometric mean of the corresponding quantity for bands I and II.

It is clear from the data of Table 1 that χ increases as the molecular weight of the dye component increases. The dye molecular weight enters Eq. 4 via the relation

$$v' = (M_4/M_3)v \quad (5)$$

where M_3 is the average mass of a DNA base pair and v is the number of moles of dye bound per mole of base pairs. Combining this with Eq. 4 it is seen that as M_4 increases, $\Delta\theta$ and therefore χ should increase roughly linearly if all of the other variables can be considered constant. This statement corresponds to a physical picture in which increasing the dye mass (M_4) increases the size (both mass and volume) of each dye "cork" without changing the number of dye "corks"

(constant ν 's) or the degree of hydration (constant Γ 's). The actual data are shown in Fig. 3 which indicates that such a simple picture is reasonably accurate.

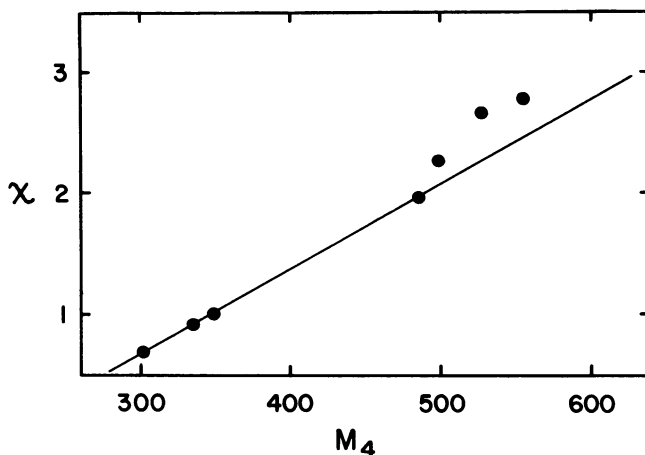


Fig. 3. The enhancement factor χ vs. the mass of the dye component. The "equilibrium" values of χ (11 days of centrifugation) are shown. The straight line is a least squares fit to the first four points.

However, a more careful analysis of the data shows that the rate of increase of χ with M_4 is much steeper than expected on this basis by over a factor of 2. The most likely reason for this enhanced resolution is that a significant increase in $\Delta\Gamma'$ occurs. There are two reasons for expecting that $\Delta\Gamma'$ should be larger for the larger dyes. First, the larger $\Delta\theta$ for these dyes means that there is a larger water activity difference between the two bands. Second, as the dye mass increases the buoyant density for both bands decreases. This is seen as a general upward movement of the bands in Fig. 1. This moves the bands into a region of water activity where the degree of hydration is becoming a very steep function of the water activity²⁰ and thus the two bands may become hydrated to a very different degree.

Combining these effects, which increase $\Delta\Gamma'$, with the dye gradient effect, which increases $\Delta\nu$, and with the direct

linear increase of $\Delta v'$ with M_4 , we can make a qualitative prediction of the shape of the plot of χ versus M_4 . The initial linear increase of χ with M_4 will curve upwards and increase rapidly as $\Delta \Gamma'$ becomes very large (due to the rapid increase of Γ' at higher water activities) and as Δv increases due to the increasingly steep dye gradient. For some very large value of M_4 a maximum value of χ will occur since the hydration of both DNA's will become very large and the density difference will decrease. This corresponds to an increase in the denominator of Eq. 4. Numerical computation of the buoyant separation $\Delta \theta$ using the parameters of Bauer and Vinograd⁵ indicates that considerable further improvement in the resolution of the dye buoyant density system is possible.

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